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# Hypoxia inducible factors in liver disease and hepatocellular carcinoma: Current understanding and future directions

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## Summary

Hypoxia inducible transcription factors (HIFs) activate diverse pathways that regulate cellular metabolism, angiogenesis, proliferation, and migration, enabling a cell to respond to a low oxygen or hypoxic environment. HIFs are regulated by oxygen-dependent and independent signals including: mitochondrial dysfunction, reactive oxygen species, endoplasmic reticular stress, and viral infection. HIFs have been reported to play a role in the pathogenesis of liver disease of diverse aetiologies. This review explores the impact of HIFs on hepatocellular biology and inflammatory responses, highlighting the therapeutic potential of targeting HIFs for an array of liver pathologies.

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## Introduction

Oxygen is vital for all living cells and transport around the body by the vasculature is regulated by the metabolic requirements and functional status of the tissue or organ [1]. The liver has a unique anatomical and functional role that affects its physiology and oxygen homeostasis [2]: receiving highly oxygenated blood via the hepatic artery and oxygen-depleted blood in the hepatic portal vein. This directional blood flow towards the central vein of the

lobule creates a physiological oxygen gradient, resulting in oxygen pressure (pO<sub>2</sub>) of 60–65 mmHg (~8%) in the periportal area to 30–35 mmHg (~4%) in perivenous areas of the parenchyma [3,4]. The resulting oxygen gradient associates with liver zonation, a phenomenon where hepatocytes show distinct functional and structural heterogeneity across the parenchyma (Fig. 1) [5–7]. Defining relative terms such as 'normoxia' and 'hypoxia' depends on the normal oxygen tension to which an organ is exposed. It is important to note that, despite the variable oxygen tensions in the liver, a hypoxic response is not observed in the normal healthy liver [8,9]. However, modest changes in oxygen tension that occur during viral hepatitis, metabolic disorders, steatohepatitis, inflammation and carcinogenesis are sufficient to promote a hypoxic response that stabilizes HIFs [3,10–12].

## Key Points

- Hypoxia inducible factors (HIFs) regulate the transcription of a wide range of genes involved in cellular metabolism, inflammation, angiogenesis and proliferation
- Low oxygen tension, inflammation and viral infection regulate HIF expression and associated transcriptional activity and are implicated in several liver diseases, including metabolic disorders, steatohepatitis, viral hepatitis and hepatocellular carcinoma
- Targeting HIFs has therapeutic potential for treating inflammatory liver pathologies

## Oxygen-dependent regulation of HIFs

Cells adapt to low oxygen through a concerted transcriptional response [13,14] regulated by HIFs, a group of proteins belonging to the PAS family (period circadian protein-aryl hydrocarbon receptor nuclear translocator-single minded protein) [15]. HIFs regulate numerous signalling events by binding specific DNA sequences known as hypoxia responsive elements (HREs) in target genes, resulting in their increased or decreased transcription [15,16]. There are three HIF transcription factors (HIF-1, HIF-2, and HIF-3) that act as heterodimers comprising an alpha and beta

Keywords: Hypoxia; Viral hepatitis; Inflammation; HIF; Liver.

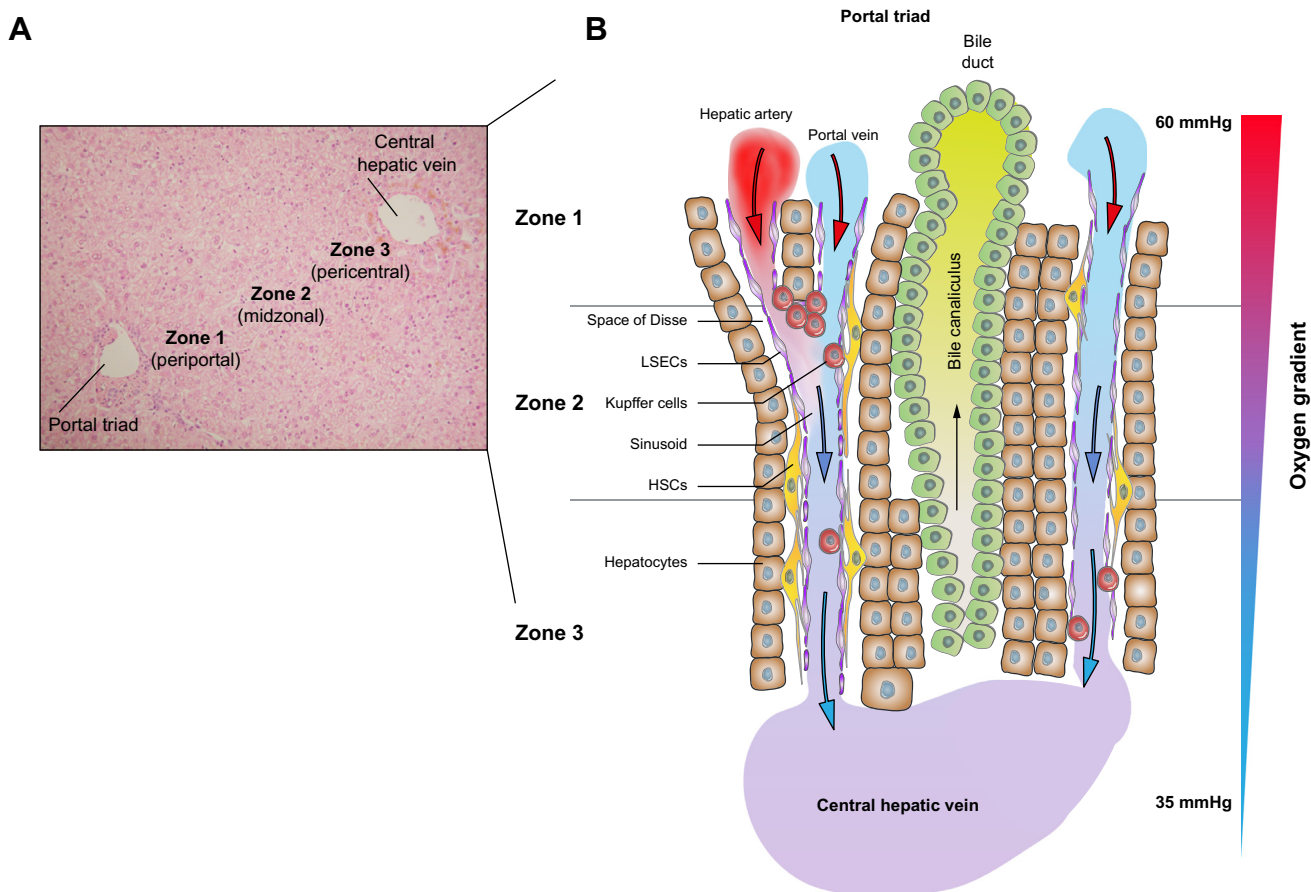
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Abbreviations: HIFs, hypoxia inducible transcription factors; pO<sub>2</sub>, oxygen pressure; HREs, hypoxia responsive elements; PHD, prolyl hydroxylase domain-containing protein; FIH, factor inhibiting HIF; pVHL, von Hippel-Lindau tumour suppressor; mTOR, mechanistic target of rapamycin; CBP, CREB binding protein; PI3K, phosphatidylinositol-3-kinase; I/R, ischemia-reperfusion; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor-beta; PDGF, platelet derived growth factor; HBx, HBV encoded protein X; HK2, hexokinase 2; LDHA, lactate dehydrogenase A; CAFs, cancer associated fibroblastic cells; LPA, lysophosphatidic acid.





**Fig. 1. Structural organization of the liver.** (A) Haematoxylin and Eosin staining of the liver lobule which is divided into 3 zones and extends from the portal triad (hepatic artery, portal vein and bile duct) to the central hepatic vein. (B) Schematic shows parenchymal (hepatocytes) and non-parenchymal cells (liver sinusoidal endothelial cells [LSECs], hepatic stellate cells [HSCs] and Kupffer cells). Blood enters the liver via the portal artery and vein (zone 1/periportal) and traverses the liver lobule via the sinusoids, incoming blood is rich in oxygen and nutrients, which depletes as the blood travels to zone 3 or the pericentral region. Arrows (red to blue) depict the oxygen gradient across the lobule. The black arrow shows the direction of the bile flow.

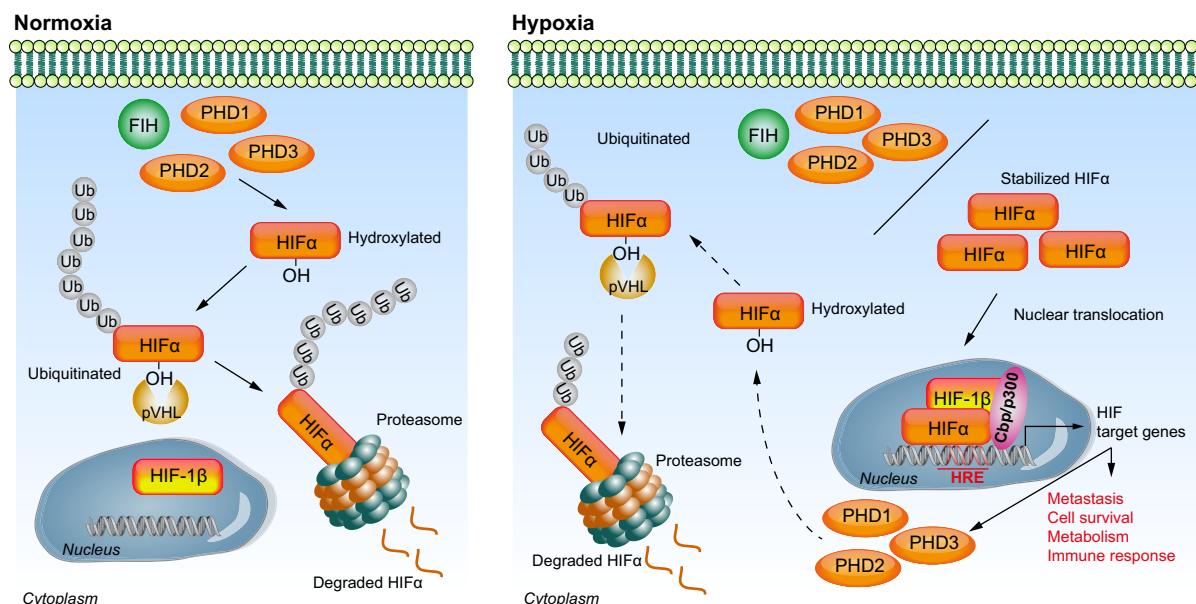
subunit. The alpha subunit is regulated via oxygen-induced proteolytic degradation, whereas the beta subunit is constitutively expressed. Although stable under hypoxia, under 'normal' oxygen tensions HIF- $\alpha$  subunits are rapidly degraded by the hydroxylation of target prolyl residues by prolyl hydroxylase domain-containing proteins (PHD1–3) [17,18]. Liver specific PHD2 deletion results in stable HIF-1 $\alpha$  expression, whereas deleting PHD3 stabilizes HIF-2 $\alpha$ , highlighting differences in HIF-regulation. Hydroxyprolyl residues are recognised by the von Hippel-Lindau (pVHL) E3 ubiquitin ligase, that polyubiquitylates the HIF- $\alpha$  subunit, targeting it for proteasomal degradation (Fig. 2). Under hypoxia, PHD activity is reduced leading to stable HIF- $\alpha$  subunit expression and binding to its regulatory elements [17] through its N-terminal and C-terminal transactivation domains. HIFs associate with the CREB binding protein (CBP) to form a transcriptional activator complex. During extended periods of low oxygen, as observed during pathological conditions, a HIF-1 $\alpha$  dependent feedback loop increases PHD expression, leading to a reactivation of HIF-1 $\alpha$  hydroxylation and degradation [19]. Thus, HIF-1 $\alpha$  expression can represent an acute response to low  $pO_2$ , whereas HIF-2 $\alpha$  levels may increase over time in hypoxia and play a role during chronic hypoxia [20].

It is important to consider another oxygen-sensitive hydroxylase, factor inhibiting HIF (FIH), which regulates HIF-1 $\alpha$

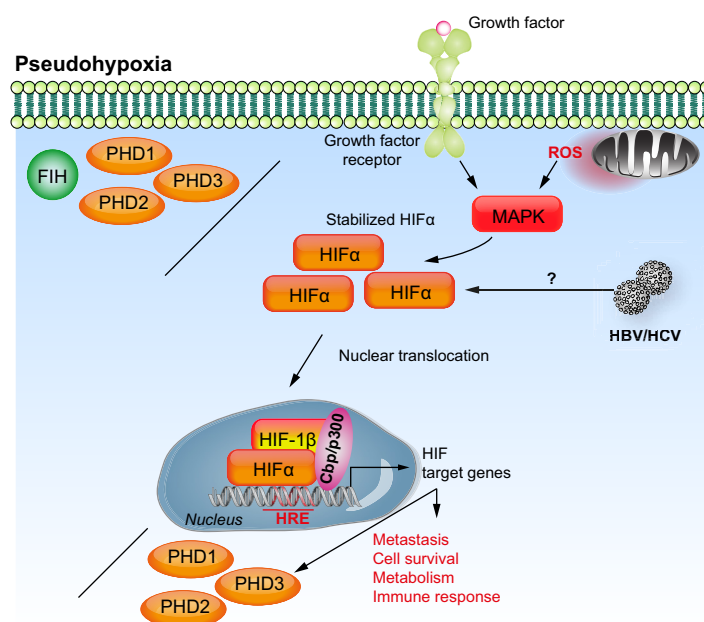
expression. FIH hydroxylates an asparaginyl residue in the C-terminal transactivation domain of HIF-1 $\alpha$  (N803 in humans), but not HIF-2 $\alpha$ , inhibiting the binding of the heterodimer HIF-1  $\alpha$  to its transcriptional coactivator p300 [21]. Although PHDs have a reported low affinity for oxygen (apparent  $K_m$  *in vitro* of 230–250  $\mu M$  – a little over 21%  $O_2$ ), the  $K_m$  of FIH is 90  $\mu M$  (~8%) [22,23]. This has significant implications for the signature of genes transactivated by hypoxia, as FIH limits the activity of the C-terminal transactivation domain of HIF-1 $\alpha$  but not the N-terminal domain [24]. In this way, cells can exhibit a biphasic HIF-1 $\alpha$ -dependent transcriptional profile: a PHD-inactivation dependent profile under moderate hypoxia, and a PHD and FIH-inactivation dependent profile under more severe hypoxia.

### Oxygen-independent regulation of HIFs

A variety of oxygen-independent signalling events and cellular stress can stabilize HIF $\alpha$  subunits in the presence of oxygen – a phenotype known as 'pseudohypoxia' (Fig. 3). Cell surface receptors, such as G protein-coupled receptors and receptor tyrosine kinases promote HIF-1 $\alpha$  mRNA translation and transactivational activity. In the phosphatidylinositol-3-kinase (PI3K) pathway, binding of a growth factor (e.g., insulin-like growth factor 1) to



**Fig. 2. Oxygen dependent HIF signalling.** Under normal oxygen tension (normoxia), the cellular oxygen sensors prolyl hydroxylases (PHD1–3) and factor inhibiting HIF (FIH) hydroxylate specific residues of HIF $\alpha$  subunits (HIF-1 $\alpha$  and 2 $\alpha$  for PHDs and HIF-1 $\alpha$  for FIH). Hydroxylated HIF $\alpha$  is recognized by the von Hippel-Lindau (pVHL) E3 ubiquitin ligase that polyubiquitinates HIF $\alpha$  resulting in proteasomal degradation. Under low oxygen (hypoxia) PHD and FIH activity is inhibited resulting in stable HIF $\alpha$  expression and nuclear translocation where it dimerizes with its beta subunit. With the help of co-activators, including Cbp/p300, the HIF complex acts as a transcription factor by binding to specific DNA sequences defined as hypoxia responsive elements (HREs), activating the transcription of genes involved in an array of signalling events including tumour metastasis, cell survival, metabolism and immune functions. The HIF $\alpha$  signalling pathway is self-regulatory, nuclear HIF-1 $\alpha$  promotes PHD expression resulting in a negative feedback loop that ensures the pathway is not constitutively active.



**Fig. 3. Oxygen independent HIF signalling.** HIF $\alpha$  can be constitutively expressed irrespective of oxygen tension due to loss of PHD and FIH function, a state defined as pseudohypoxia. This can occur as a result of virus infection or aberrant kinase signalling. For example, binding of a growth factor to its cognate receptor activates the MAPK pathway that stabilizes HIF $\alpha$ . Similarly mitochondrial dysfunction can promote reactive oxygen species (ROS) production that acts on MAPK to stabilize HIF $\alpha$ . Hepatitis B and C viruses stabilize HIF-1 $\alpha$  under normoxia via an unknown mechanism.

its cognate receptor activates PI3K that stimulates the downstream serine/threonine kinase Akt and the mechanistic target of rapamycin (mTOR), providing a link between the microenvironment and HIF signalling, since mTOR activity is dependent on the local concentration of amino acids. In addition, growth fac-

tors can activate ERK and p70S6K1, an essential factor required for HIF-1 $\alpha$  mRNA translation. In addition to activating p70S6K1, ERK can stimulate MAPK-interacting protein that activates the translation initiator factor eIF4E together with mTOR by inhibiting the 4E-binding protein (4E-BP) (reviewed in [25,26]).



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Changes in nutrient availability can directly affect the levels of HIF $\alpha$  proteins. Since both PHDs and FIH are dependent on  $\alpha$ -ketoglutarate for their activity, conditions that result in low concentrations of this 2-oxo acid are likely to increase HIF transcriptional activity. Finally, mitochondrial dysfunction through the production of reactive oxygen species (ROS) from the electron transport chain can stabilize HIF $\alpha$  subunits by inactivating PHD enzymatic activity [27]. ROS levels are increased during inflammation through various mechanisms, including the activity of NADPH oxidase, as well as downstream of calcium release in response to endoplasmic reticular stress [28–31].

The HIF system is regulated by a number of interconnected signalling mechanisms *in vivo*. In pathological conditions affecting the liver where HIFs are stabilized, the underlying mechanism may alter with time, resulting in prolonged HIF-1 $\alpha$  expression. For example, during ischaemia, an acute reduction in blood flow stabilizes HIF-1 [32]. However, upon reperfusion, the influx of ROS can inactivate the PHDs [27], leading to oxygen-independent HIF expression. This is followed by an increase in cellularity through immune cell infiltration and vascular remodelling that alters supply whilst increasing oxygen and nutrient demand, allowing HIF signalling through yet another means. It is likely that, at the lower end of the physiological gradient of oxygen in the liver (i.e.  $\sim 4\%$  O<sub>2</sub>), small variations in tissue pO<sub>2</sub> or non-hypoxic pathways to regulate HIFs become highly significant. This review explores the impact of HIFs on hepatocellular biology and immunity; highlighting how hypoxia or pseudohypoxia induction of HIFs contributes to a wide range of liver pathologies and may provide new therapeutic targets.

### HIFs in liver ischemia-reperfusion

Ischemia-reperfusion (I/R) mediated injury of the liver occurs during hepatic resection and organ preservation prior to transplantation and is a major factor in graft dysfunction [33]. During ischemia, loss of oxygen and nutrients lead to a precipitous drop in adenosine triphosphate levels that lead to a loss of membrane function and metabolic dysfunction. However, the cellular damage that occurs following restoration of the blood supply (reperfusion) via ROS-mediated oxidation of cellular proteins and lipids can have significant functional deficits, if not cell death. Although perfusion of the liver with completely anoxic fluid results in uniform ischemia throughout the lobule, ischemia due to reduced flow was heterogeneous with the pericentral liver showing signs of ischemic metabolic responses whilst the remaining liver was unperturbed [34]. Evidence from a number of organ studies suggest that HIFs play an important role in protecting the liver from I/R injury [35–37]. HIF activation lies downstream of a number of well-described protective pathways, including adenosine, nitric oxide and AKT signalling [35,38], and its activity supports oxygen-independent ATP generation as well as upregulation of 'cell preservation systems', such as anti-oxidant and anti-apoptotic proteins that allow for cell survival during and directly post ischemia.

### HIFs and fatty liver disease

Fatty liver disease or hepatic steatosis, the excessive accumulation of macro- or microvesicular cytoplasmic lipid droplets in

hepatocytes, is a major health concern due to its contribution to obesity, type 2 diabetes and cardiovascular disease (reviewed in [39]). Clinically it is categorized into alcoholic fatty liver disease (AFLD) and non-alcoholic fatty liver disease (NAFLD) both of which can lead to fibrosis, cirrhosis and HCC [40]. Several reports demonstrate that rats fed a continuous ethanol diet show liver hypoxia [8,41,42], although the direct contribution of HIF-1 to alcoholic liver injury is unknown. Studies with rat hepatocytes show enhanced *de novo* lipogenesis in perivenular cells, coupled with increased esterification of exogenous fatty acids into cellular and very low density lipoprotein lipids [43], indirectly supporting a role for low oxygen to regulate hepatic lipid metabolism. The literature supports a role for HIFs to regulate hepatic lipogenesis [3,44,45] and recent investigations have sought to define the relative contributions of HIF-1 and 2 $\alpha$ .

The role of HIF-1 $\alpha$  in steatohepatitis is the subject of conflicting reports. Hepatocyte specific *Hif1a* knockout mice fed an ethanol diet showed increased triglyceride and lipid accumulation via inhibiting the Hif-1 $\alpha$  target gene, differentiated embryo chondrocyte gene 1, (*Dec1*). Overexpression of *Dec1* reversed the effects of alcohol on liver biology, supporting a positive regulatory role for Hif-1 $\alpha$  regulated genes in protecting mice from AFLD [46]. However, Nath and colleagues reported that activating *Hif1a* in a cre-lox mouse model induced hepatocyte steatosis and increased triglyceride levels with ethanol feeding, whereas *Hif1a* deletion prevented ethanol-induced lipid accumulation [9]. The reasons for the disparity between these studies are not clear and may reflect differences in the murine models used; however, the findings of Nath *et al.* are in line with recent data showing that *Hif2* knockout adult mice develop steatosis [47]. In contrast, Kim and colleagues reported that activating *Hif1a* or *Hif2a* in the murine liver had a minimal effect on lipid accumulation. Activating both *Hif1a* and *Hif2a* promoted macrovesicular lipid accumulation [48], however, the relationship of this phenotype to human diseases characterized by steatosis remains to be elucidated.

Genetic inactivation of *Vhl/Hif1a* in transgenic mice, which results in HIF-2 $\alpha$  specific responses, induced steatohepatitis with impaired fatty acid  $\beta$ -oxidation and increased lipid droplets in hepatocytes. Importantly, *Hif2a* inactivation decreased steatohepatitis. These data highlight a potential role for HIF-2 $\alpha$  to deregulate hepatic lipid homeostasis [49]. Indeed, *Vhl* disruption in mice augmented hepatic lipid accumulation in a HIF-2 $\alpha$  dependent manner and genome array studies demonstrate increased transcription of genes associated with fatty acid synthesis and uptake [11]. These studies highlight HIF-2 $\alpha$  as a potential therapeutic target for treating steatohepatitis. However, HIF-2 $\alpha$  perturbation of hepatic lipogenesis is intertwined with improved insulin signalling in liver specific *Phd3* knockout mice, that exhibit stable hepatic HIF-2 $\alpha$  [50]. These observations were corroborated by Wei and colleagues who reported a link between Hif-2 $\alpha$  expression in murine liver and increased hepatic insulin sensitivity via induction of insulin receptor substrate 2 [51]. In addition, the authors showed improved glucose tolerance and insulin signalling in diabetic and non-diabetic mice following treatment with vascular endothelial growth factor (VEGF) inhibitors. VEGF inhibition induced local hypoxia by limiting sinusoidal vascularization resulting in HIF-2 $\alpha$  expression [51]. Taken together, these data highlight the multiple roles HIF-2 $\alpha$  plays in the liver, where expression can both ameliorate diabetes and potentiate dyslipidaemia. Further studies are required to define the mechanisms by which HIFs modulate hepatic lipid homeostasis.

## HIFs and inflammation

Chronic hepatitis of diverse aetiologies is characterized by immune cell infiltration that promotes liver damage, however, the impact of varying oxygen levels across the liver parenchyma on immune cell function is not known. Inflammation is well recognised to induce a shift in metabolic supply-and-demand ratios that lead to localized tissue hypoxia, inducing cell-type dependent HIF-transcriptional activities that regulate pro-inflammatory and anti-inflammatory responses [52]. A recent study demonstrated that HIF-1 $\alpha$  upregulates TLR4 expression in macrophages, suggesting that hypoxic stress at inflammatory sites enhances innate cellular responses to bacterial pathogens [53]. Hypoxic macrophages show increased expression of IFN-gamma [54], MHC-II and co-stimulatory molecules that may promote antigen presenting capacity and immune synapse formation leading to increased T cell cytokine production [55]. A study of myeloid-specific *Hif1* knockout mice reported an essential role for HIF-1 in dendritic cells that defined interferon and T cell activation [56]. Larbi *et al.* reported altered CD3/CD28-dependent T cell proliferation and a switch in the respiratory pathway toward glycolysis at low oxygen tensions, suggesting that activation thresholds, proliferation and susceptibility to apoptosis will differ under varying oxygen levels [57]. Two recent reports highlight a positive regulatory role for hypoxia to induce FOXP3 expression in human and murine mononuclear cells, leading to an increased frequency and potency of regulatory T cells (Tregs) to suppress effector cell proliferation [58,59]. This observation is consistent with reports showing that interleukin-8 producing FOXP3<sup>+</sup>CD4<sup>+</sup> regulatory T cells are enriched in the liver of subjects with chronic HCV and HBV infection [60–62]. Collectively, these studies highlight the multi-factorial role for HIFs to link the innate and adaptive immune systems and illustrate how hypoxia-dependent changes in metabolic signals can induce an anti-inflammatory program, providing new therapeutic opportunities for the treatment of chronic liver inflammation.

Liver fibrosis is characterised by the excessive deposition of extracellular matrix (ECM) proteins such as type I collagen in the liver parenchyma, representing a wound-healing response to persistent or repeated injury [63]. Modification of the parenchymal vasculature can promote regions of hypoxia; of note the pattern of fibrosis varies according to the underlying disease. HIF-1 $\alpha$  expression can activate hepatic stellate cells (HSCs) and fibroblasts to differentiate into myofibroblasts that proliferate and migrate to injured areas where they secrete ECM [64–67]. *In vivo* studies using bile duct ligated mice, an animal model of liver fibrosis, show increased Hif-1 $\alpha$  expression 3 days after surgery whereas *Hif1a*-deficient ligated mice show a significant reduction in fibrogenic mediators [66].

Macrophages are recruited to the liver following injury to sites of inflammation where they secrete transforming growth factor-beta (TGF- $\beta$ ) and platelet derived growth factor (PDGF) that can activate HSCs and promote fibrogenesis [68,69]. Nuclear HIF-1 $\alpha$  was detected in macrophages, fibroblasts and hepatocytes in liver biopsies collected from subjects with primary biliary cirrhosis and primary sclerosing cholangitis [70]. Together, these data support a potential role for macrophage expressed HIFs in the development of hepatic fibrosis, however, the acquisition of a hypoxic phenotype by macrophages in the context of fibrosis is unknown. A potential positive role for HIF-2 $\alpha$  in liver fibrosis was supported by experimental studies in genetically manipu-

lated mice, where liver specific disruption of *Vhl* enhanced the transcription of pro-fibrogenic genes in a HIF-2 $\alpha$  dependent manner, as confirmed by whole genome microarray analysis. In contrast, pro-fibrogenic gene expression was significantly reduced in mice carrying a *Hif2a* specific deletion [11]. Taken together, these data suggest complementary roles for HIFs in liver fibrosis.

## HIFs and viral hepatitis

It is interesting to note that both hepatitis B and C viruses stabilize HIF-1 $\alpha$  and promote a pseudohypoxic state. Despite their different replication strategies, both viruses have developed successful ways to establish chronic infection, resulting in 350 and 180 million infected subjects worldwide, respectively [71]. Infection by either virus leads to serious and progressive liver disease, including steatosis, fibrosis, cirrhosis and HCC. HBV encoded protein X (HBx) is a multifunctional protein with transcriptional activity via its interaction with nuclear transcription factors and modulation of cytoplasmic signal transduction pathways, such as RAS/RAF/MAP signalling [72]. Transgenic mice expressing the HBx gene develop hepatic pathological changes including adenomas and malignant carcinomas [73]. *In vitro* studies suggest that oncogenicity of HBx is mediated via HIF-1 $\alpha$  expression, resulting in enhanced cell invasion and proliferation [74,75]. A recent study demonstrated that mutations commonly found in the HBx gene enhanced HIF-1 $\alpha$  expression and transcriptional activity [76], suggesting differences between infecting viral genotypes that may predict HCC tumour pathogenesis. Although a growing body of evidence suggests that HBx stabilizes HIF-1 $\alpha$ , the majority of studies have been performed with ectopic protein expression as opposed to infectious virus, reflecting the limited infectivity of full-length HBV genomes *in vitro*. Nevertheless, investigating HBx activity in the context of an infected cell is a worthwhile endeavour and highlights the need to develop robust culture models to propagate HBV *in vitro*.

Studies with HCV demonstrate a virus-induced pseudohypoxic response [10,77,78], showing stable HIF-1 $\alpha$  expression under normoxic conditions. Furthermore, hypoxia promotes HCV replication, while inhibiting HIF-1 $\alpha$  activity reduced viral replication [12,79]. Current understanding of the role that HIF-1 $\alpha$  plays in the HCV lifecycle is limited. However, one may hypothesize that HIF-dependent changes in hepatocyte permeability and metabolism favours viral transmission and replication, respectively. We previously reported that hepatocyte polarity restricts HCV entry *in vitro* and the virus overcomes this barrier by promoting HIF-dependent transcriptional activation of *VEGF* that depolarizes hepatocytes and aids viral dissemination [80,81]. *VEGF* is a well-characterized HIF-1 $\alpha$  regulated gene that plays an essential role in angiogenesis and HCV infection, and is associated with elevated neoangiogenesis [77], suggesting a key role for HIF-regulated genes in potentiating the virus lifecycle [12,80].

The development of high-throughput metabolomics has provided new insights into how viruses modulate host metabolism [82,83]. Metabolic profiling of HCV infected cells revealed a shift from an energy consuming to energy conserving phenotype, promoting the survival of infected cells [84]. Given the role of HIF-1 $\alpha$  in regulating glucose metabolism, HCV stabilization of HIF-1 $\alpha$  may have a positive effect on virus replication via the induction of a transformed metabolic phenotype. Ripoli *et al.*, investigated the effect of constitutive HIF-1 $\alpha$  activity on hepatocyte metabolism during HCV infection [78], reporting a reduction in

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mitochondrial oxidative phosphorylation and increased glycolytic enzyme expression. These findings highlight a metabolic shift that is reminiscent of aerobic glycolysis and provides insights into the effect of viral infection on host bioenergetics. Moreover, these observations suggest that HIF-1 $\alpha$ -dependent cellular reprogramming may promote HCV-associated HCC pathogenesis.

### HIFs and hepatocellular carcinoma

HCC is the most common primary liver malignancy, with an estimated 750,000 new cases and 695,000 deaths per year, rating third in incidence and mortality in the world. The major risk factor for HCC is chronic liver disease with a high frequency of hepatitis B and C infections [85]. Whilst incidence and mortality for other cancers is declining, this tumour represents an increasing significant public health problem. Despite advances in HCC treatment, most patients die within one year of diagnosis largely due to recurrence and metastases. Hypoxia is a prominent feature of solid tumours as a result of their defective vascularization and intense metabolic activity, associating with poor prognosis and resistance to chemotherapeutic agents/radiation [86]. HIF mediates expression of genes involved in every step of HCC metastasis including EMT [87], invasion of the extracellular matrix, intravasation, extravasation and secondary growth of metastases. HIF-1 $\alpha$  expression in HCC is a negative prognostic factor for clinical outcome after surgery [88] and is associated with metastatic potential [89,90]. The impact of HBV and HCV stabilization of HIFs in HCC pathogenesis is not known.

Solid tumours showing high levels of glycolysis and hypoxia-mediated transcriptional responses reinforce and strengthen the glycolytic phenotype by upregulating almost every enzyme in this pathway, including hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA). Glycolysis has been reported to fuel hypoxic solid tumours such as hepatocellular carcinoma (HCC), where increased HK2 expression stimulates the proliferation of malignant cells. Moreover, inhibiting HK2 expression in a murine HCC model increased tumour cell apoptosis and limited tumour growth [91,92]. The production of lactate from pyruvate is regulated by LDHA and its knockdown has been shown to suppress tumour growth and metastasis *in vivo* and *in vitro* [93]. Together these reports suggest a role for glucose metabolism in HCC metastasis and HIF-1 $\alpha$  signalling is likely to play an essential role. Indeed, HIF-1 $\alpha$  stabilization may be one of the drivers of aerobic glycolysis, a phenotype observed in many tumour types [94].

Ma and colleagues demonstrated reduced gluconeogenesis in malignant hepatocytes in a murine HCC tumour model and showed that restoring gluconeogenesis with a synthetic steroid inhibited hepatocellular tumour growth [95]. Since HIF-2 $\alpha$  expression has been reported to reduce hepatic gluconeogenesis [96], it is tempting to speculate a positive regulatory role for HIF-2 $\alpha$  in HCC pathogenesis. However, recent data showing that overexpression of HIF-2 $\alpha$  in murine HCC models inhibits tumour growth, highlight the need for further studies to address the role of this transcription factor in HCC metastasis [97].

### HIFs and HCC microenvironment

The HCC microenvironment comprises tumour cells within the extracellular matrix and stromal cells that include: angiogenic cells, immune cells and cancer associated fibroblastic cells (CAFs).

There is a growing body of evidence on the role stromal cells play in defining cancer progression and response to therapies, particularly in breast, lung and pancreatic carcinomas [98]. Alterations within the microenvironment, in particular in stromal fibroblasts, may influence tumour initiation in adjacent epithelia and promote progression. Moreover, the microenvironment plays an important role in chemoresistance [99,100] and drug delivery [101]. Targeting stromal cells to abrogate their tumour-supporting role represents an attractive therapeutic strategy. However, our current understanding of how stromal cells respond to low oxygen and the various paracrine pathways that are elicited to promote HCC growth are poorly defined [102]. CAFs represent one of the most dominant cell types in most tumours, however, there are limited studies on this cell type in HCC. Lin *et al.* reported that two human CAF cell lines promote HCC by upregulating the expression of cytokines and growth factors (*APLN*, *CCL2*, *CCL26*, *CXCR4*, *IL6*, *MUC1*, *LOXL2*, *PDGFA*, *PGK1*, *VEGFA*) related to proliferation, migration, invasion and angiogenesis [103]. A recent study showed that HCC-derived tumour cells secrete lysophosphatidic acid (LPA) that activates peritumoral fibroblasts to adopt a CAF phenotype and accelerates HCC progression [104]. The authors demonstrated that inhibiting LPA blocked the transdifferentiation of myofibroblasts and tumour progression in murine models, providing an exciting new therapeutic target. Given a number of reports showing that LPA activates PI3K and stabilizes HIF-1 $\alpha$  in adipocytes [105], colon tumour cells [106] and PC3 prostate cancer cells [107], it is tempting to speculate a HIF-1 $\alpha$  dependent pathway in HCC.

HCC is frequently associated with large numbers of lymphocytes, including tumour-specific CD8 cells, regulatory T cells, NKT and NK cells. Several immunoregulatory mechanisms have been implicated in the suppression of anti-tumour immunity, including expression of pro-apoptotic molecules such as PD-1 on tumour cells, recruitment of regulatory T cells, immunosuppressive myeloid derived cells (MDSCs) and inactivation of NK responses by soluble NKG2D ligands (MICA/B) [59,108,109]. PD-1 ligand was recently reported to be a direct target of HIF-1 $\alpha$  [110], suggesting that the simultaneous blockade of PD-L1 and HIF-1 $\alpha$  may represent a novel approach to treat HCC. Doedens *et al.* recently reported that HIFs influence the expression of transcription, effector and costimulatory-inhibitory molecules of viral specific cytotoxic T cells, highlighting new strategies to promote the clearance of viruses and tumours [111]. Thus, HIF-1 $\alpha$  can exert direct effects on tumour and vascular cells that prime selective chemokine-mediated recruitment of suppressive and pro-angiogenic T-cell subsets. Our current understanding of how hypoxic conditions affect innate immune cells and their role in HCC tumourigenesis is poorly understood and further research is required to increase our understanding of the role HIFs plays in anti-HCC immunity [112].

### HIFs and HCC treatment

HCC has dual causes of morbidity and mortality, namely the cancer and the underlying chronic liver disease. The majority of patients present with disease too advanced for surgical resection and require liver transplantation. A small percentage of patients are suitable for trans-arterial chemoembolisation, a procedure that exploits the vascularity of HCC tumour nodules, whilst this may provide a modest increase in survival, the disease progresses and treatment-associated hypoxia promotes revascularisation,



invasion and metastasis [113]. Anti-angiogenic agents that target HIF-regulated VEGF are used as first and second line treatments for several cancers, including HCC. The kinase inhibitors sorafenib and sunitinib that target VEGF receptors have been approved for the treatment of HCC and gastrointestinal stromal tumours, respectively. Recent randomised phase 3 placebo-controlled trials demonstrate a survival advantage for the multi-targeted kinase inhibitor sorafenib in patients with advanced HCC [114]. However, the effects are modest with a median improvement in overall survival of 2 to 3 months with no clinical or molecular biomarkers to identify patients most likely to benefit. A number of randomised trials of other VEGF-targeted drugs (including sunitinib and brivanib) have failed to demonstrate any further survival benefit in either the first or second line setting and most patients die within one year of diagnosis, largely due to further metastases [115]. Therapy-induced tumour hypoxia has been observed in response to diverse treatments, including radiotherapy [116,117], chemotherapy [118,119], anti-angiogenic and vascular disrupting agents [120]. Current data suggest that resistance to VEGF inhibitors associates with a more invasive/metastatic tumour phenotype and activation of HIF-dependent angiogenic pathways.

The high failure rate of phase 3 trials for HCC is forcing the scientific community to optimize trial design and to consider molecular tumour profiling to select patients most likely to respond to therapy [121]. The search for molecular predictors of response is becoming standard practice in clinical oncology research and relies on the concept of 'oncogenic addiction' that is based on *a priori* knowledge of the specific molecular alterations for tumour progression on an individual basis [122]. Unlike other solid tumours (e.g., lung, colon or breast) there is limited information on oncogenic addiction loops in HCC. Considerable research efforts have focused on the molecular profiling of HCC tumours with the goal of identifying gene signatures that predict disease outcome following treatment [123–126]. To date, there has been limited concordance between studies, that may reflect HCC tumour heterogeneity [127]. Van Malenstein *et al.* reported a seven-gene hypoxic gene signature that correlated with poor prognosis in HCCs, however, the authors defined hypoxic tumour gene signatures by comparing mRNA profiles to an *in vitro* control sample where HepG2 hepatoblastoma cells were propagated under 2% oxygen for 72 h. From our experience, HIF expression and transcriptional activity is optimal between 24 and 48 h post-hypoxic treatment and declines significantly by 72 h, limiting the conclusions of this study. More recently, Nault *et al.* reported a HCC five-gene score, associated with patient survival following liver resection in different clinical settings, including the identification of four new genes *RAN*, *TAF9*, *RAMP3*, and *HN1* which reflect signalling pathways associated with HCC prognosis. It will be interesting to see whether any of these genes are transcriptionally regulated by HIFs and how this new biomarker will perform in the clinical setting to stratify patients for therapy.

### HIF as a therapeutic target

Several *in vivo* studies highlight the value of targeting the HIF pathway to inhibit tumour progression [128] and to improve the efficacy of VEGF-targeted therapies by modulating the hypoxic tumour microenvironment. Targeting HIFs can limit the unwanted effects of therapy-induced tumour hypoxia observed with  $\gamma$ -radiation [116,129] and anti-angiogenic agents, resulting

in significantly improved treatment efficacy in pre-clinical models [120]. A number of pharmacological agents that target HIF either directly or indirectly are in pre-clinical studies or clinical trials, mostly as novel means of treating advanced cancers [118,130]. The HIF-1 $\alpha$  antagonist EZN-2968 is a locked nucleic acid antisense oligonucleotide that specifically downregulates HIF-1 $\alpha$  mRNA and protein expression. *In vivo* studies show a specific and long-lasting down-modulation of HIF-1 $\alpha$  and VEGF in the liver of mice and anti-tumour effects on human xenografts [131]. We previously reported that inducing PHD enzyme activity with derivatives of alpha-ketoglutarate represent a novel method to target hypoxic areas of tumours [132,133]. Unlike therapies that differentiate between HIF isoforms, this would reduce signalling through both HIF-1 $\alpha$  and 2 $\alpha$ , which may be more tumouricidal than approaches that only target one. Agents that inhibit reactive oxygen species generation, such as superoxide dismutase mimetics, have also been shown to decrease HIF levels [134]. Other less direct mechanisms of reducing HIF-1 transcriptional activity include the use of mTOR inhibitors such as the rapalogs, inhibitors of AKT activity or dual specificity mTOR/PI3K inhibitors like NVP-BE225 [135] that target other important signalling pathways in parallel with the HIF axis. These studies highlight the therapeutic potential of targeting HIFs for the treatment of HCC, however, our review of the currently available literature suggests that targeting HIFs may improve an array of liver pathologies including steatohepatitis and viral hepatitis.

### Concluding remarks

Recent studies highlight a role for HIFs in the pathogenesis of chronic liver disease and HCC. Much of our current understanding is based on murine models and it will be important to translate these observations into man and to develop *in vitro* culture systems that incorporate hepatic oxygen levels: enabling studies to characterize the impact of oxygen tension on liver resident and infiltrating immune cell function *ex vivo*. It is interesting to note that both HBV and HCV induce a pseudohypoxic cellular phenotype. However, this observation is not unique to these hepatotropic viruses, with a number of other viruses including Epstein-Barr virus and Cytomegalovirus inducing a cellular hypoxic response [136,137]. Viral hijacking of the HIF-pathway is likely to provide significant benefits to many steps in the viral life cycle, however, it may also provide a novel therapeutic target to limit viral replication and associated pathologies.

### Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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